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<b>(21) International Application Number:</b> PCT/US99/15568  <b>(22) International Filing Date:</b> 9 July 1999 (09.07.99)  <b>(30) Priority Data:</b> 60/092,416                      10 July 1998 (10.07.98)                      US  <b>(71) Applicant (for all designated States except US):</b> U.S. MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES [US/US]; Department of the Army, 504 Scott Street, Frederick, MD 21702 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LEE, John, S. [US/US]; 1807 Elizabeth Court, Hagerstown, MD 21740 (US). PUSHKO, Peter [US/US]; 917 Seminole Road, Frederick, MD 21701 (US). PARKER, Michael, D. [US/US]; Apartment T-13, 1001 Carroll Parkway, Frederick, MD 21701 (US). SMITH, Jonathan, F. [US/US]; 6936 Eylers Valley Flint Road, Sabillasville, MD 21780 (US). WELKOS, Susan, L. [US/US]; 1594 Dockside Drive, Frederick, MD 21701 (US).  <b>(74) Agents:</b> HARRIS, Charles, H.; United States Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702 (US) et al.		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> ANTHRAX VACCINE		
<b>(57) Abstract</b>  Using the nontoxic PA protein from <i>B. anthracis</i> , a method and composition for use in inducing an immune response which is protective against anthrax in subjects is described.		

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**TITLE OF THE INVENTION**

Anthrax Vaccine

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**FIELD OF THE INVENTION**

This invention relates to vaccines for bacterial toxins from *Bacillus anthracis*.

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**INTRODUCTION**

Anthrax is a disease caused by the sporulating bacteria *Bacillus anthracis*. Humans working with animal products are at risk from contracting anthrax. Areas such as Iran, Turkey, Iraq, Pakistan, and sub-Saharan Africa are hyperendemic for anthrax, although the organism can be found in most areas of the world. Anthrax manifests disease in three different ways. Inhalation, gastrointestinal, and cutaneous anthrax result from inhaling spores, ingesting spores in contaminated meat, or contacting spores in an open wound, respectively. Untreated inhalation or gastrointestinal anthrax has a case fatality rate of essentially 100 percent while cutaneous anthrax has a case fatality rate of up to 25 percent. Previous research has shown that the protective antigen (PA) produced by *B. anthracis* can protect mice from anthrax. Even though the Anthrax vaccine is FDA licensed, reactogenicity is mild to moderate.

Therefore, there is a need for an efficacious vaccine for anthrax useful for protecting humans.

#### SUMMARY OF THE INVENTION

5       The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against infection with anthrax.

10       In this vaccine strategy, a gene coding for a protein of interest is cloned into a VEE virus vector in place of the VEE virus structural genes; the result is a self-replicating RNA molecule, a replicon, that encodes its own replicase and transcriptase functions,

15       and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is

20       packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in trans. Since the helper RNAs lack packaging signals necessary for further propagation, the resulting VEE replicon particles (VRPs) which are produced are

25       infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is ultimately killed by the infection, but does not

30       produce any viral progeny (Pushko et al., 1997, *Virology* 239, 389-401).

      The PA gene which contains a prokaryotic secretory signal and the entire 83 kDa coding sequence (Welkos et al., 1988, *Gene* 69, 287-300), was inserted

into the VEE replicon vaccine vector (Figure 1) and have demonstrated high level expression of this bacterial protein in eukaryotic cells in culture. Mice, either the C57BL/6 strain or the A/J strain, inoculated with VRP containing the PA-replicon produced high specific antibody titers and were protected from developing anthrax when challenged subcutaneously with *B. anthracis*.

Therefore, it is one object of the present invention to provide a VEE virus replicon vector comprising a VEE virus replicon and DNA fragments encoding the PA protein from *B. anthracis*.

It is another object of the present invention to provide a self replicating RNA comprising the VEE virus replicon and any of the *B. anthracis* fragments described above.

It is another object of the present invention to provide infectious VEE virus replicon particles produced from the VEE virus replicon RNA described above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against *B. anthracis* infection comprising VEE virus replicon particles containing any of the *B. anthracis* fragments described above or a combination of different VEE virus replicons each having a different *B. anthracis* fragment.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

**Figure 1.** Diagram of the protective antigen (PA) replicon constructs. The PA replicons are similar to

the full-length VEE RNA except that the open reading frame encoding the VEE structural proteins was replaced with the PA genes.

**Figure 2.** Western blot of BHK cell lysates showing expression of anthrax protective antigen from recombinant VEE replicons. a) uninfected cell lysate; b) recombinant expression product from *B. anthracis*; c) infected cell lysate.

**Figure 3A and 3B.** ELISA titers for mice (A, C57Bl/6 mice; B, A/J mice) immunized with PA-VRP. GMTs only includes those mice that survived challenge. a) Received 0.2 ml on day 0 and 28. b) Received  $10^7$  iu on day 0 and 28. c) Received  $10^7$  iu PA-VRP on day 0, 28, 56, and 84; C57Bl/6 mice that died, titer = 100; A/J mice that died, titer = 400. d) Received  $10^7$  iu PA-VRP on day 0, 28, and 56; C57Bl/6 mice that died, titers = 400, 100, 100; A/J mice that died, titers = 102400, 100, 1600. e) Received  $10^7$  iu PA-VRP on day 0, and 28; C57Bl/6 mice that died, GMT = 1313; A/J mice that died, GMT = 1766. Mice were challenged 28 days after last inoculation with 18-32 LD<sub>50</sub> Sterne strain for C57Bl/6 and 12-25 LD<sub>50</sub> Sterne strain for A/J mice.

**Figure 4.** Schematic representation of constructs containing PA.

#### DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

**Replicon.** A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site.

Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA *in trans*. This is typically done with two helpers also called defective helper RNAs. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then be inoculated into animals similar to parent virus. The replicon

particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nonstructural proteins and the product of the heterologous gene cloned in place of the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which the structural proteins genes are replaced with a gene from an immunogen of interest, in this invention, the *B. anthracis* proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the glycoprotein and capsid proteins of the VEE virus.

**Subject.** Includes both human, animal, e.g., horse, cattle, donkey, monkey, pig, dog, guinea pig, mouse, hamster, avian e.g., chicken, pheasant or turkey, fish and other marine animals, and insects such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a VEE replicon and a DNA sequence encoding the non-toxic protective antigen (PA) from *B. anthracis*. The sequence for PA has been determined and has been deposited in GenBank at accession no. M22589. The PA sequence encodes a prokaryotic secretory signal in addition to the entire sequence encoding the 83 Kd PA (SEQ ID NO:1). Other nucleic acid sequences related to this invention include sequences wherein the secretory signal has been removed (MAT-PA) (SEQ ID NO:2), or replaced with other secretory signals known to people in the art such as the tissue plasminogen activator (TPA) secretory signal resulting in a DNA



fragment encoding TPA-PA (SEQ ID NO:3). Nucleic acid sequences included in this invention are sequences encoding the active form of PA, a 63 kDa protein, termed PA63 (SEQ ID NO:4). In addition, isolated  
5 nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the *B. anthracis* proteins described and specified in  
10 SEQ ID NO:5 (TPA-PA), SEQ ID NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63). Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate  
15 variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli*).

Nucleic acid molecules of the present invention  
20 may be in the form of RNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the  
25 sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For  
30 example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or  
35 substantially) DNA molecules in solution. Isolated

RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such  
5 molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences  
10 of at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is  
15 position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the *B. anthracis* polypeptides described above. Variants may occur naturally, such as a  
25 natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occurring variants may be produced by known mutagenesis techniques. Such  
30 variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid  
35 substitutions, deletions, or additions. Especially

preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of the *B. anthracis* polypeptides disclosed herein or portions thereof.

- 5 Also preferred in this regard are conservative substitutions.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The  
10 vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The  
15 cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences; or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with  
20 ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. When the DNA sequences described above are in a  
25 replicon expression system, such as the VEE replicon described above, the proteins can be expressed *in vivo*. The DNA sequence for any of the *B. anthracis* proteins described above can be cloned into the multiple cloning site of a replicon such that  
30 transcription of the RNA from the replicon yields an infectious RNA containing the *B. anthracis* protein or proteins of interest. Use of helper RNA containing sequences necessary for encapsulation of the viral transcript will result in the production of viral  
35 particles containing replicon RNA which are able to

infect a host and initiate a single round of replication resulting in the expression of the *B. anthracis* proteins. Such replicon constructs include those listed in Table 1.

5 Table 1

Replicon

Plasmid Name

expresses

10	p3014-TPA-PA	TPA signal, 5 amino acids of C terminal end of prokaryotic secretory signal, and the 83kDa mature protein.
	p3014-PA	29 amino acid prokaryotic secretory signal, and the 83kDa mature protein secreted by <i>B. anthracis</i>
15	p3014-MAT-PA	83kDa mature protein
	p3014-PA63	63kDa protein (minus 20kDa N-terminal end that is naturally cleaved off by furin proteases at host cell membrane surface)
20	p3014-sub-domains	subdomain #1: from nucleotide 1891 to 2391; subdomain #2: from nucleotide 2392 to 2658; subdomain #3: from nucleotide 2659 to 3351; subdomain #4: from nucleotide 3352 to 3669; subdomain #5: from nucleotide 3670 to 4098 (Subdomains are numbered according to Welkos et al., 1988, supra, determined on subdomains present in the crystal structure, Petosa, et al., 1997, Nature 385, 833-838).
25		
30		
35		

The VEE constructs containing anthrax proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

40 In another embodiment, the present invention relates to RNA molecules resulting from the transcription of the constructs described above. The RNA molecules can be prepared by *in vitro* transcription using methods known in the art and  
 45 described in the Examples below. Alternatively, the

RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current  
5 Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for example, as a nucleic acid vaccine, or to transfect cells along with RNA from helper plasmids, one of which expresses VEE  
10 glycoproteins and the other VEE capsid proteins, as described above, in order to obtain replicon particles.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection,  
15 electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. et al. (Eds), Wiley & Sons, Inc. All documents cited herein supra and infra are hereby  
20 incorporated in their entirety by reference thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for  
25 example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences  
30 when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions,

and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of protein of the invention, such as glutathione S-transferase. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as baby hamster kidney (BHK) cells, MRC-5 cells and vero cells, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase

expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein or polypeptide specified as amino acid sequence in SEQ ID NO:5 (TPA-PA), SEQ ID NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63).

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

The recombinant or fusion protein can be used as a vaccine for immunity against anthrax infection or as a diagnostic tool for detection of bacillus anthracis. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit anthrax or *B. anthracis* proteins, such as host

proteins or chemically derived agents or other proteins which may interact with *B. anthracis* proteins of the present invention to inhibit its function. A method for testing the effectiveness of an anti-anthrax drug or agent can for example be the rat anthrax toxin assay (Ivins et al. 1984, *Infec. Immun.* 52, 454-458 and Ivins et al. 1986) or a skin test in rabbits for assaying antiserum against anthrax toxin (Belton and Henderson, 1956, *Br. J. Exp. Path.* 37, 156-160).

In another embodiment, the present invention relates to an anthrax vaccine comprising one or more replicon particles derived from one or more replicons encoding one or more *B. anthracis* proteins or polypeptides as described above. The present invention relates a method for providing immunity against anthrax said method comprising administering one or more replicon particles containing any combination of the *B. anthracis* proteins to a subject such that a protective immune reaction is generated.

Vaccine formulations of the present invention comprise an immunogenic amount of a replicon particle, resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about  $10^2$  to  $10^7$  per dose is suitable, more or less can be used depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.



Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be use in any combination effective to elicit an immunogenic response in a subject. Generally, the nucleic acid vaccine administered may be in an amount of about 1-5 ug of

nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

**Plasmids.** Construction of the VEE replicon, capsid (C-) helper, and glycoprotein (GP-) helper plasmids was previously described (Pushko, 1997, supra). The PA gene from nucleotide 1804 to 4098 (SEQ ID NO:1) (Welkos et al., 1988, supra) was cloned into the VEE replicon plasmid as a BamHI/BamHI fragment utilizing a shuttle vector. TPA-PA (SEQ ID NO:3), MAT-PA (SEQ ID NO:2), and PA63 (SEQ ID NO:4) were PCR amplified using forward and reverse primers which also contained the ClaI recognition site. The PCR products were agarose gel purified and ligated into the T/A vector PCR2.1 (Invitrogen, Inc.). The PA genes, after

digestion of pCR2.1-PA's with ClaI, were ligated into the VEE replicon plasmid. The Lassa nucleocapsid replicon (Lassa N-replicon) was constructed as previously described (Pushko, 1997, supra) and used as  
5 a negative control replicon.

**Production of VRP.** Plasmid templates for the PA-replicons, C-helper, GP-helper, and the Lassa N-replicon were linearized by digestion with NotI at a unique site downstream from the coding sequences, and  
10 capped run-off transcripts were prepared *in vitro* using T7 RNA polymerase. Packaging of the replicons into VEE replicon particles (VRPs) was accomplished by electroporating the replicon RNA and the two helper RNAs into BHK cells. VRPs were harvested between 20  
15 and 27 hours after transfection and purified from cell culture supernatants by ultracentrifugation through a discontinuous sucrose gradient (20%). After reconstituting the pelleted VRP in 1/50 volume phosphate buffered saline, the VRPs were stored at -  
20 70°C.

**Analysis of expression products and titration of VRP.** Subconfluent monolayers were infected with PA-VRP's (either TPA-PA, PA, MAT-PA, or PA63) or Lassa N-VRP (m.o.i. = 2) or cell suspensions  
25 were electroporated with replicon RNA. Cells were harvested at approximately 20-24 hours and expressed proteins were separated by SDS-PAGE. Visualization of PA proteins was accomplished using a chemiluminescence western blot assay and antibodies specific for each  
30 protein. Titration of VRPs was accomplished by infecting subconfluent monolayers with increasing dilutions of purified VRP. Antigen positive cells were visualized in an indirect immunofluorescence assay using a monoclonal antibody specific for each protein,

or in a direct immunofluorescence assay using an FITC-conjugated monkey anti-Lassa serum.

**Immunization of mice.** Mice were inoculated 1 to 4 times at 28 day intervals with  $10^5$  to  $10^7$  infectious units (iu) of either PA-VRP or Lassa N-VRP (negative control). Positive control mice for the anthrax study were inoculated subcutaneously with 0.2 ml of anthrax vaccine at 28 day intervals. Serum for ELISA was obtained 2 days before each inoculation and 3 days before challenge.

**Enzyme-linked immunosorbent assay (ELISA).** Microtiter plates were coated with protective antigen (gift from Joe Farchaus, Bacteriology Division) (1 ug/ml) in PBS and allowed to absorb overnight at 4°C. Four fold serum dilutions in blocking buffer were applied to the plates and incubated at 37°C for 1 hour. After washing, an anti-mouse secondary antibody (HRP conjugated) was added to the plate and incubated for an additional hour at 37°C. After washing, bound antibody was detected colormetrically using ABTS as a substrate.

**Challenge of mice.**

Anthrax challenge: C57BL/6 or A/J mice were challenged subcutaneously with 12-32 LD<sub>50</sub> *B. anthracis* (Sterne) 31 days after the last inoculation.

**Example 1**

**Packaging and expression of PA-replicon**

The PA-replicon was efficiently packaged into VRPs using the double helper system. Stock solutions contained about  $10^8$  infectious units of purified PA-VRP per milliliter. No replication competent VEE virus was detected in any of the preparations. A western blot of cell lysates, generated from BHK cells infected with PA-VRP, demonstrates a product of

approximately 85 kDa (Figure 2). Because the PA-VRP expression product contains a 29 amino acid prokaryotic secretory signal, which is cleaved from the standard, the PA product from the BHK cells appears larger than the PA standard.

#### Example 2

##### **Protection against challenge with *B. anthracis***

The results of the animal studies demonstrated that PA from *B. anthracis* could be used to immunize and protect mice from a lethal challenge of *B. anthracis* (Sterne strain). Although the Sterne strain is attenuated in some animals, it is virulent in mice. Controls in the experiment included mice vaccinated with the current human vaccine (anthrax vaccine absorbed, AVA) which protected all mice, and mice immunized with the Lassa N-replicon which expressed an antigen irrelevant to anthrax protection which failed to protect the mice. Figure 3A shows ELISA titers and survival for C57Bl/6 mice inoculated with two to four doses of PA-VRP. Seventy percent of the C57Bl/6 mice which received three inoculations of the PA-VRP were protected whereas 90% of the C57Bl/6 mice which received four inoculations were protected from an otherwise lethal challenge of *B. anthracis* (Sterne strain). The geometric mean of the serum ELISA titers to PA of C57Bl/6 mice given four doses of the PA-replicon was 150500, as compared to 264 for mice that were inoculated with the negative control replicon. Figure 3B shows ELISA titers and survival for A/J mice inoculated with two to four doses of PA-VRP. Three inoculations of the PA-VRP protected 70% of the A/J mice, whereas four inoculations protected 90% of the A/J mice from an otherwise lethal challenge of the *B. anthracis* (Sterne strain). The geometric mean of the serum ELISA titers against PA in A/J mice given four

doses of the PA-replicon was 223336 as compared to 100 for mice which were inoculated with the negative control replicon. As expected, none of the mice (from either strain) which were inoculated with the negative control replicon survived challenge with *B. anthracis* (Sterne strain).

### Discussion/Conclusion

Since VEE virus replicates in the cytoplasm of eukaryotic cells, the VEE replicon vaccine vector is a useful tool for the expression of prokaryotic genes in eukaryotic cells. Cytoplasmic expression of genes alleviates the difficulties imposed by splicing and nuclear transport of mRNA. We used the VEE replicon as a way to express the prokaryotic PA gene in eukaryotic cells and to develop a new vaccine candidate against anthrax.

The human AVA contains mostly PA protein and is presumed to protect humans by eliciting an antibody response that can neutralize the PA portion of anthrax toxin produced by invading *B. anthracis*. After neutralization of the toxin, the immune system destroys the invading bacteria. In order to model the human disease, we chose to use mice and the Sterne strain of *B. anthracis*. The Sterne strain produces anthrax toxin (encoded on the pXO1 plasmid) but lacks a capsule (encoded on the pXO2 plasmid). Mice exposed to large quantities of Sterne (ranging from  $10^3$  to  $10^8$  spores for the different mouse strains) are not able to overcome the effects of the toxin produced by the bacteria so they succumb to infection. We hypothesized that by immunizing the mice with PA, thereby inducing neutralizing antibodies to the PA

portion of the toxin produced by a challenge inoculum,  
that the mice would be protected.

A VEE replicon was constructed which expressed  
the PA gene from *B. anthracis* and was evaluated for *in*  
5 *vitro* expression, *in vivo* immunogenicity, and  
protective efficacy. C57Bl/6 and A/J mice  
demonstrated increasing antibody responses to PA when  
inoculated with increasing doses of PA-VRP. The two  
mouse strains inoculated with up to four doses of PA-  
10 VRP displayed increased protection against a lethal  
challenge with *B. anthracis* (Sterne strain). The use  
of mice and the Sterne strain of *B. anthracis* useful  
as a model system for studying the immunogenicity and  
efficacy of replicon based anthrax vaccines.

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What is claimed is:

1. A recombinant DNA construct comprising:
  - (i) a vector, and
  - (ii) at least one nucleic acid
- 5 fragment comprising any combination of *B. anthracis* proteins selected from the group consisting of PA, MAT-PA, TPA-PA, and PA63.
2. A recombinant DNA construct according to claim
- 10 1 wherein said vector is an expression vector.
3. A recombinant DNA construct according to claim
- 1 wherein said vector is a prokaryotic vector.
- 15 4. A recombinant DNA construct according to claim
- 1 wherein said vector is a eukaryotic vector.
5. The recombinant DNA construct of claim 1
- wherein said vector is a VEE virus replicon vector.
- 20 6 The recombinant DNA construct according to
- claim 5 wherein said construct is p3014-PA.
7. The recombinant DNA construct according to
- 25 claim 5 wherein said construct is p3014-TPA-PA.
8. The recombinant DNA construct according to
- claim 5 wherein said construct is p3014-MAT-PA.
- 30 9. The recombinant DNA construct according to
- claim 5 wherein said construct is p3014-PA63.
10. Self replicating RNA produced from a
- construct chosen from the group consisting of: p3014-
- 35 PA, p3014-TPA-PA, p3014-MAT-PA, and p3014-P63



11. Infectious alphavirus particles produced from packaging the self replicating RNA of claim 10.

5        12. A pharmaceutical composition comprising infectious alphavirus particles according to claim 11 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

10       13. A host cell transformed with a recombinant DNA construct according to claim 5.

14. A host cell according to claim 13 wherein said host cell is prokaryotic.

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15. A host cell according to claim 13 wherein said host cell is eukaryotic.

20       16. A method for producing *B. anthracis* protein comprising culturing the cells according to claim 14 under conditions such that said DNA fragment is expressed and said protein is produced.

25       17. A method for producing *B. anthracis* protein comprising culturing the cells according to claim 15 under conditions such that said DNA fragment is expressed and said protein is produced.

30       18. A vaccine for anthrax comprising viral particles containing one or more replicon RNA encoding any combination of *B. anthracis* proteins chosen from the group consisting of PA, PA-MAT, TPA-PA, and PA63.

35       19. A pharmaceutical composition comprising the self replication RNA of claim 14 in an effective

immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

20. A pharmaceutical composition comprising one  
5 or more recombinant DNA constructs chosen from the  
group consisting of p3014-PA, p3014-TPA-PA, p3014-MAT-  
PA, and p3014-P63 in a pharmaceutically acceptable  
amount, in a pharmaceutically acceptable carrier/and  
or adjuvant.

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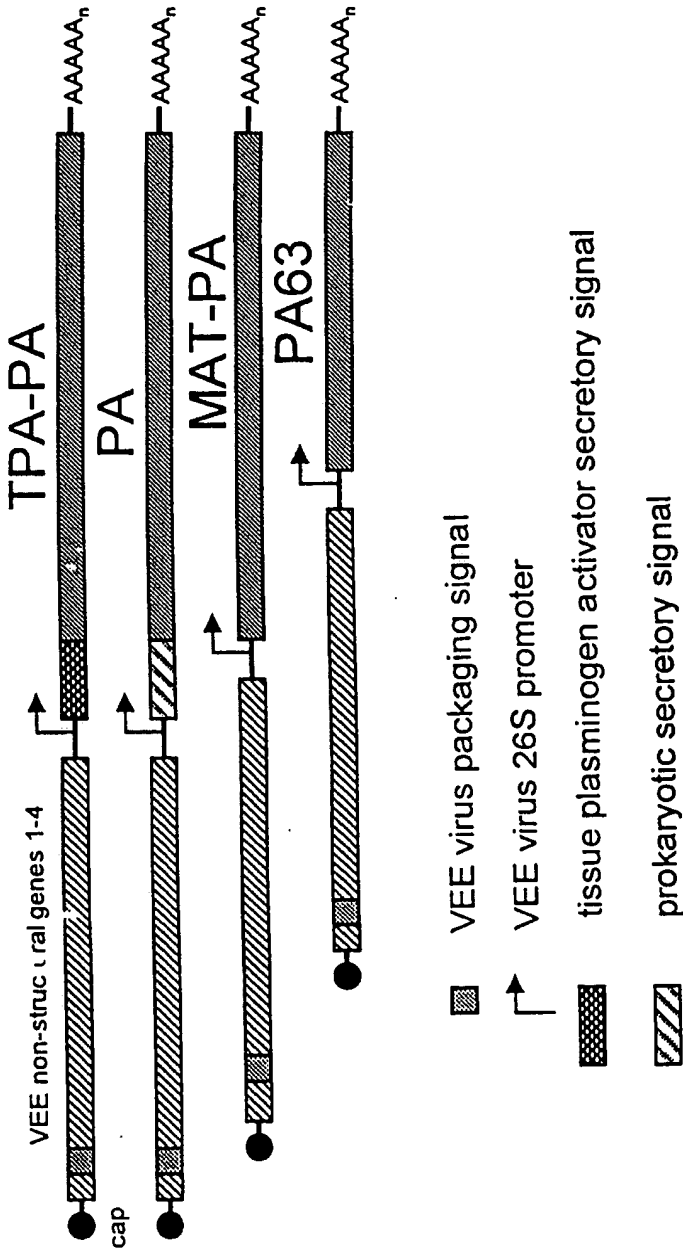


Figure 1. Diagram of the protective antigen (PA) replicon constructs. The PA replicons are similar to the full-length VEE RNA except that the open reading frame encoding the VEE structural proteins was replaced with the PA genes.

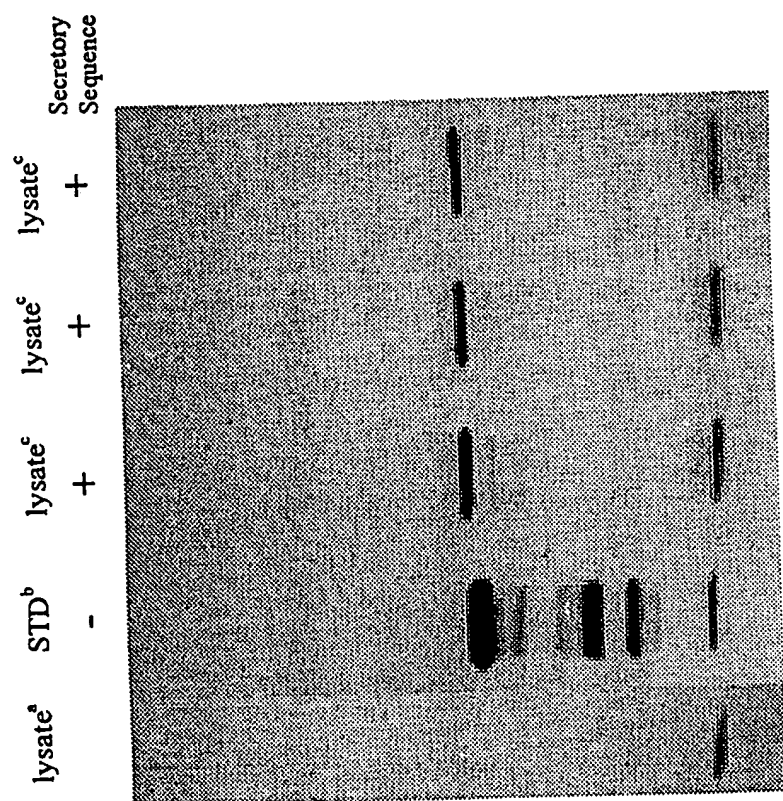


Figure 2. Western blot of BHK cell lysates showing expression of anthrax protective antigen from recombinant VEE replicons. a) uninfected cell lysate; b) recombinant expression product from *B. anthracis*; c) infected cell lysate.

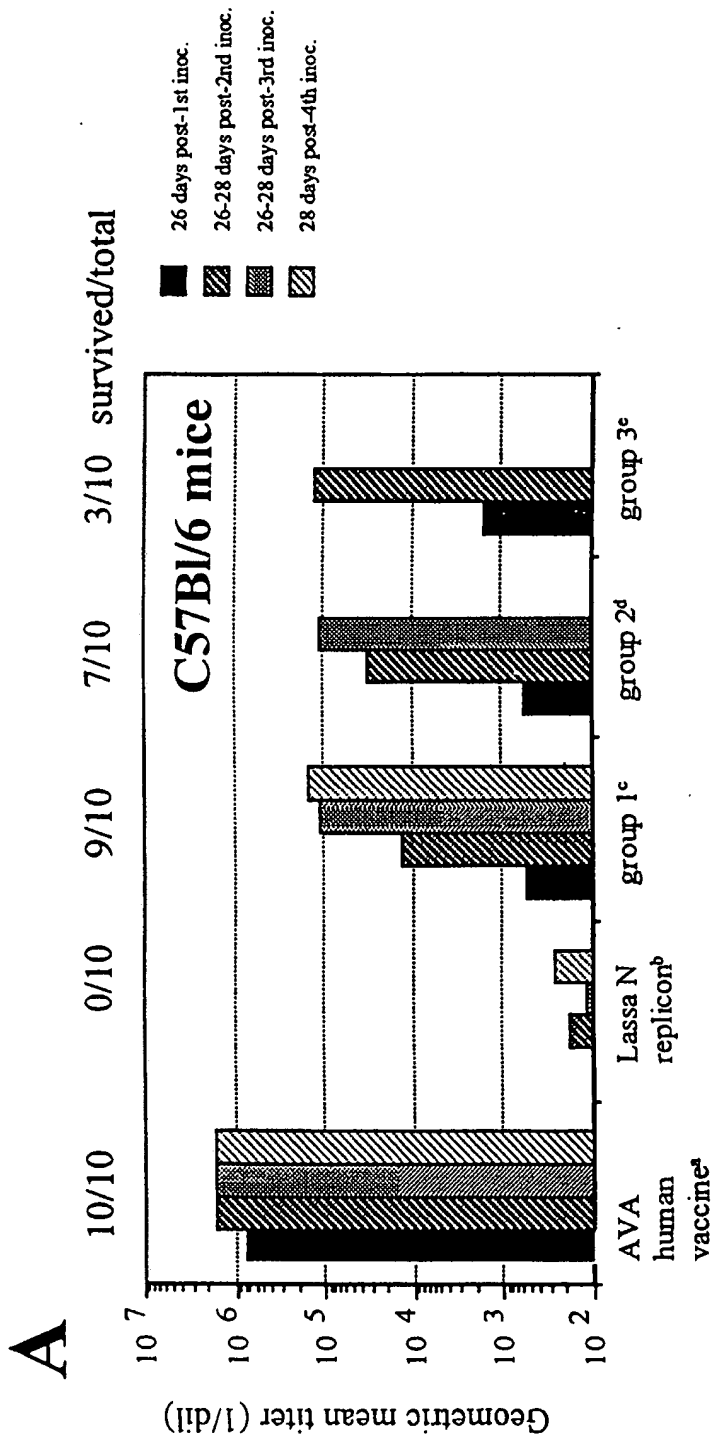
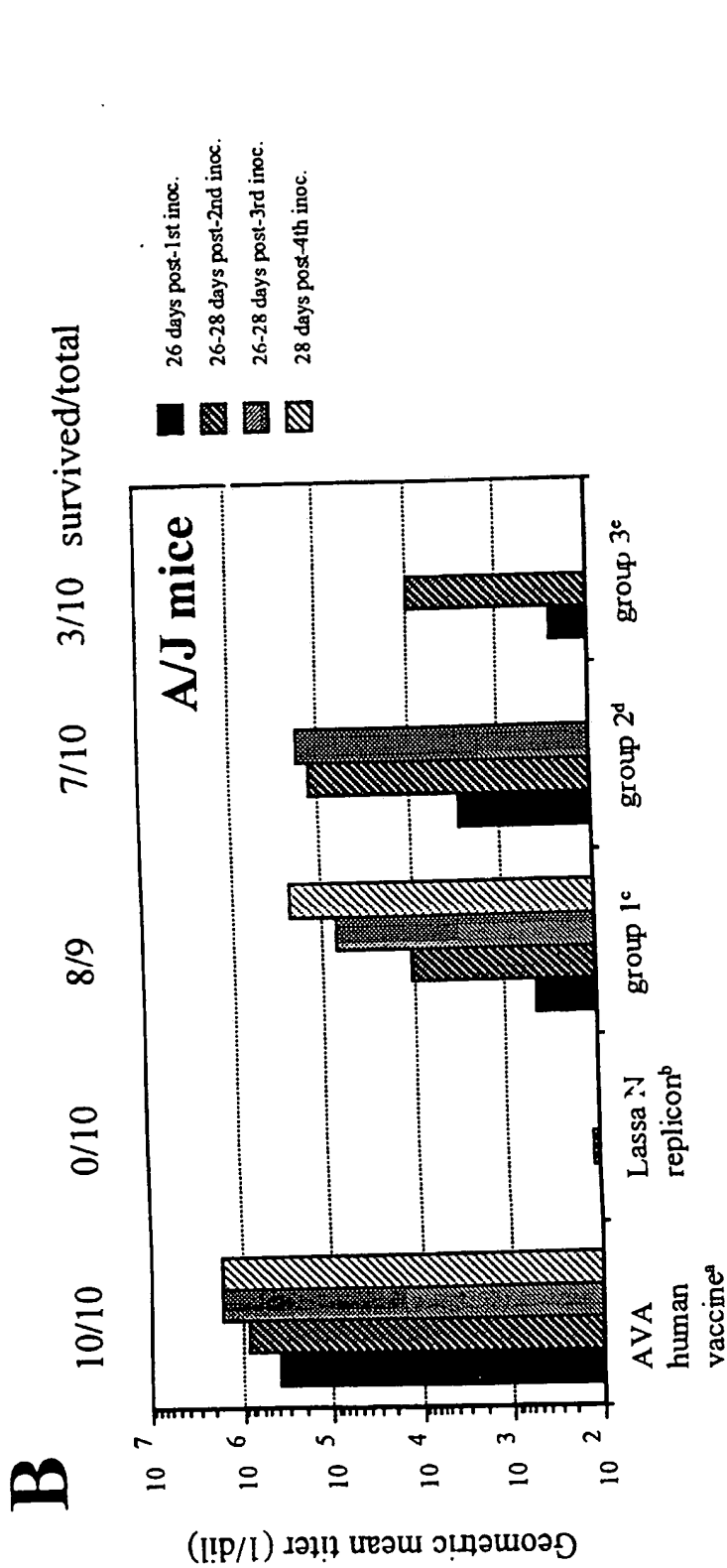
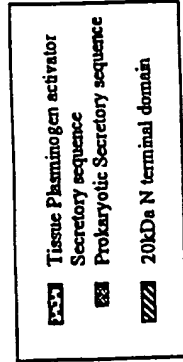


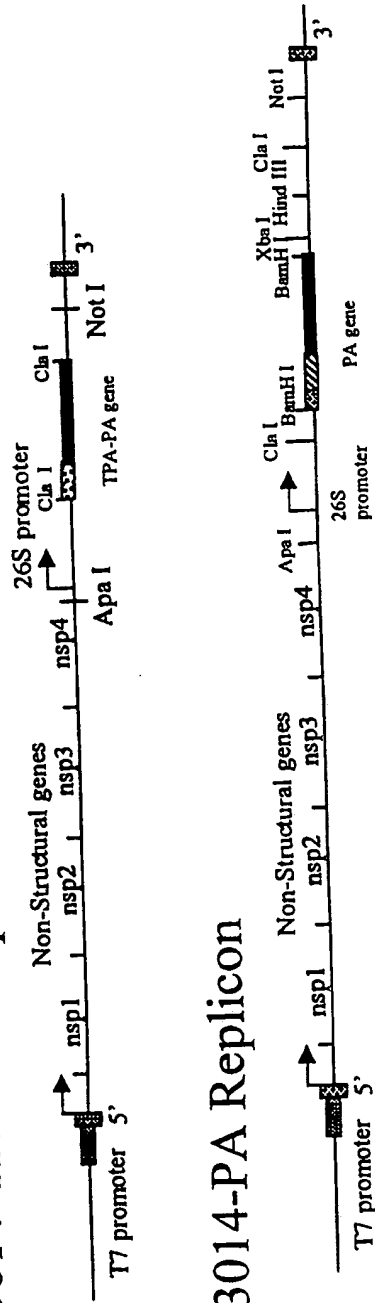
FIGURE 3



**Figure 3.** ELISA titers for mice immunized with PA-VRP. GMTs only includes those mice that survived challenge. a) Received 0.2 ml on day 0 and 28. b) Received 10<sup>7</sup> iu on day 0 and 28. c) Received 10<sup>7</sup> iu PA-VRP on day 0, 28, 56, and 84; C57Bl/6 mice that died, titer = 100; A/J mice that died, titer = 400. d) Received 10<sup>7</sup> iu PA-VRP on day 0, 28, and 56; C57Bl/6 mice that died, titers = 400, 100, 100; A/J mice that died, titers = 102400, 100, 1600. e) Received 10<sup>7</sup> iu PA-VRP on day 0, and 28; C57Bl/6 mice that died, GMT = 1313; A/J mice that died, GMT = 1766. Mice were challenged 28 days after last inoculation with 18-32 LD<sub>50</sub> Sterne strain for C57Bl/6 and 12-25 LD<sub>50</sub> Sterne strain for A/J mice.



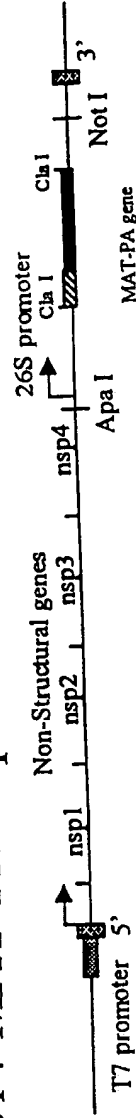
### p3014-TPA-PA Replicon



### p3014-PA Replicon



### p3014-MAT-PA Replicon



### p3014-PA63 Replicon

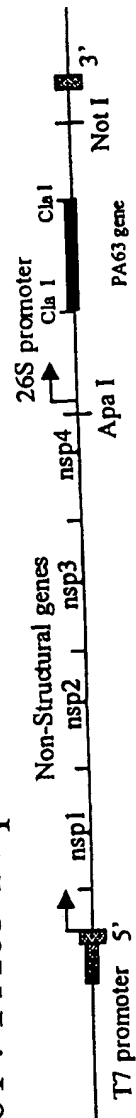


Figure 4

## TPA-PA amino acid sequence

SEQ ID NO: 15

1 MDAMKRGLLCCVLLCGAVFVSASEVIQAEVKQENRLLNESESS  
 44 SQGLLGYYFSDLNFQAPMVVTSSTTGDLSIPSELENIPSENQYFQSAIWSGFIKVKK  
 102 SDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD  
 160 FKLWYTDSDQNKKEVSSDNLQLPELKQKSSNSRKRSTAGPTVPDRDNDGIPDSLEV  
 218 EGYTVDVKNKRTFLSPWISNIHEKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV  
 276 SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTSEVHG  
 334 NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNAN  
 392 IRYVNTGTAPIYNNVLPPTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL  
 450 NAQDDFSSTPITMNYNQFLELXKQLRLDQVYGNIAFYNFENGRVVRVDTGSNWSE  
 508 VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNEPN  
 566 GNLYQYQKIDITEFDNFQDQTSQNTKQNLAEELNATNIYTVLDKIKLNAKMNILIRDKR  
 624 FHYDRNNIAVGADSVVKEAHREVINSSTEGLLNIDKDIRKILSGYIVEIEDTEGLK  
 682 EVINDRYDMLNISSLRQDGKTFIDFKKYNDKPLIYNPNYKVVVAVTKENTIINPS  
 706 ENGDTSTNGIKILIFSKKGYEIG

## TPA-PA gene sequence

SEQ ID NO: 3

1 atgg atgcaatgaa gagagggctc tgctgtgtgc tgctgtgtg tggagcagtc  
 55 ttcggtttcgg ctacgcagggt gattcaggca gaagttaaac aggagaaccg gttattaaat  
 115 gaatcagaat caagttccca ggggttacta ggatactatt ttagtgattt gaattttcaa  
 175 gcacccatgg tggttacctc ttctactaca ggggatttat ctattcctag ttctgagtta  
 235 gaaaaatatt catcgaaaa ccaatatatt caatctgcta ttgggtcagg atttatcaaa  
 295 gttagaaga gtgatgaata tacatttgct acttccgctg ataatcatgt aacaatgtgg  
 355 gtagatgacc aagaagtgtat taataaagct tctaattcta acaaatcag attagaaaaa  
 415 ggaagattat atcaataaaa aattcaatat caacgagaaa atcctactga aaaaggattg  
 475 gatttcaagt tgtactggac cgattctcaa aataaaaaag aagtgatttc tagtgataac  
 535 ttacaattgc cagaattaaa acaaaaatct tcgaactcaa gaaaaaagcg aagtacaagt  
 595 gctggacctc cgggtccaga ccgtgacaat gatggaatcc ctgattcatt agaggtagaa  
 655 ggataacgg ttgatgtcaa aaataaaaaga acttttcttt caccatggat ttctaataat  
 715 catgaaaaga aaggattaac caaatataaa tcatctcctg aaaaatggag cagggttct  
 775 gatccgtaca gtgatttcga aaagggtaca ggacggattg ataagaatgt atcaccagag  
 835 gcaagacacc cccttgtggc agcttatecg attgtacatg tagatatgga gaatattatt  
 895 ctctcaaaaa atgaggatca atccacacag aatactgata gtgaaacgag aacaataagt  
 955 aaaaataact ctacaagtag gacacatact agtgaagtac atggaaatgc agaagtgcac  
 1015 gcgtcgttct ttgatattgg tgggagtgt tctgcaggat ttagtaattc gaattcaagt  
 1075 acggctcgaa ttgatcattc actatctcta gcaggggaaa gaacttgggc tgaaacaatg  
 1135 gggtttaata ccgctgatac agcaagatta aatgccataa ttagatatgt aaatactggg  
 1195 acggctccaa tctacaacgt gttaccaacg acttcgttag tgttaggaaa aaatacaaca  
 1255 ctccgcgaca ttaaagctaa ggaaaaccaa ttaagtcaa tacttgcacc taataattat  
 1315 tatccttcta aaaacttggc gccaatcgca ttaaatgcac aagacgattt cagttctact  
 1375 ccaattacaa tgaattacaa tcaatttctt gagttagaaa aaacgaaaca attagatta  
 1435 gatacggatc aagtatatgg gaatatagca acatacaatt ttgaaaatgg aagagtgagg  
 1495 gtggatacag gctcgaactg gagtgaagt ttaccgcaa ttcaagaaac aactgcacgt  
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 1675 tttgatttta acgaaccgaa tggaaaacta caatatcaag ggaagacat aaccgaattt  
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 1795 gcaactaaca tatatactgt attagataaa atcaaatcaa atgcaaaaat gaattttta  
 1855 ataagagata aacgttttca ttatgataga aataacatag cagttggggc ggatgagtca  
 1915 gtagttaagg aggtcatag agaagtaatt aattcgtcaa cagagggatt attgttaaat  
 1975 attgataagg atataagaaa aatattatca ggttatattg tagaaattga agatactgaa  
 2035 gggcttaaaag aagttataaa tgacagatat gatagtttga atatttctag ttacggcaa  
 2095 gatggaaaaa catttataga ttttaaaaaa tataatgata aattaccgtt atatataagt  
 2155 aatcccaatt ataaggtaaa tgtatatgct gttactaaaag aaaacactat tattaatcct  
 2215 agtgagaatg gggatactag taccaacggg atcaagaaaa ttttaattct ttctaaaaaa  
 2275 ggctatgaga taggataa



## PA amino acid sequence

SEQ ID NO: 6<sup>2</sup>

1 MGRKVLIPLMALSTILVSSTGNLEVTQAEVKQENRLLNESESS  
 45 SQGLLGYYFSDLNFOAPMVVTSSTGDLSPSELENI PSENQYFQSAIWSGFIKVIK  
 103 SDEYTFATSAONHVMTMWDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD  
 161 FKLYWTDSONKQEVISSDNQLPELKQKSSNSRKRSTASGPTVPDRNDGIPDSLEV  
 219 EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV  
 277 SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG  
 335 NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAE TMGLNTADTARLNAN  
 393 IRYVNTGTAPIYNVLPFTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL  
 451 NAQDDFSSTPITMNYNQFLELEKTKQLRLD TDQVYGNIA TYNFENGRVVRVDTGSNWSE  
 509 VLPQIQETTARIIFNGKDLNIVERRIAAVNPSDPLETTKPDMLKEALKIAFGFNEPN  
 567 GNLQYQGKDITEFDNFQDQTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKR  
 625 FHYDRNNIAVGADSVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLK  
 683 EVINDRYDMLNISSLRQDGKTFIDFKKYNDKPLYSNPYKVNVAVTKENTIINPS  
 707 ENGDTSTNGIKILIFSCKGYEIG

## PA gene sequence

- SEQ ID NO: 4

1 atgaaaa aacgaaaagt gttaatacca ttaatggcat tgtctacgat attagtttca  
 58 agcacaggta atttagaggt gattcaggca gaagttaaac aggagaaccg gttatttaaat  
 118 gaatcagaat caagttccca ggggttacta ggatactatt ttagtgattt gaattttcaa  
 178 gcacccatgg tggttacctc ttctactaca ggggatttat ctattcctag ttctgagtta  
 238 gaaaaatattc catcgaaaa ccaatatctt caatctgcta ttctggcagg atttatcaaa  
 298 gttaagaaga gtgatgaata tacatttgcct acttccgctg ataatcatgt aacaatgtgg  
 358 gtagatgacc aagaagtgat taataaagct tctaattcta acaaaatcag attagaaaaa  
 418 ggaagattat atcaataaaa aattcaatat caacgagaaa atcctactga aaaaggattg  
 478 gatttcaagt tgtactggac cgatttctcaa aataaaaaag aagtgtttc tagtgataac  
 538 ttacaattgc cagaattaaa acaaaaatct tcgaactcaa gaaaaagcg aagtacaagt  
 598 gctggaccta cggttcaga ccgtgacaat gatggaatcc ctgattcatt agaggtagaa  
 658 ggatatacgg ttgatgtcaa aaataaaaga acttttcttt caccatggat ttctaattt  
 718 catgaaaaa aaggattaac caaatataaa tcatctcctg aaaaatggag cagggttct  
 778 gatccgtaca gtgatttcga aaagggtaca ggacggattg tagatatgga gaattattt  
 838 gcaagacacc cccttgtggc agcttatccg attgtacatg tagatatgga gaattattt  
 898 ctctcaaaaa atgaggatca atccacacag aatactgata gtgaaacgag aacaataagt  
 958 aaaaataactt ctacaagtag gacacatact agtgaagtac atggaatgc agaagtgc  
 1018 gcgtcgttct ttgatattgg tgggagtgtg tctgcaggat ttagtaattc gaattcaagt  
 1078 acggtcgcaa ttgatcattc actatctcta gcaggggaaa gaacttgggc tgaacaatg  
 1138 ggtttaaata ccgctgatac agcaagatta aatgccaata tttagatatg aaatactggg  
 1198 acggtcctcaa tctacaacgt gttaccaacg acttcgtag tggtaggaaa aaatcaaca  
 1258 ctgcgcacaa tttaaagctaa ggaaaaccaa ttaagtcaaa tacttgcacc taataattt  
 1318 tatccttcta aaaacttggc gccaatcgca ttaaatgcac aagacgattt cagttctact  
 1378 ccaattacaa tgaattacaa tcaatttctt gagttagaaa aaacgaaaca attagatta  
 1438 gatacggatc aagtatatgg gaatatagca acatacaatt ttgaaaatgg aagagtggg  
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 1558 atcattttta atggaaaaga tttaaatctg gtagaaaggc ggatagcggc ggttaattct  
 1618 agtgatccat tagaaacgac taaaccggat atgacattaa aagaagccct taaaatagca  
 1678 tttggattta acgaaccgaa tggaaactta caatatcaag ggaaagacat aaccgaattt  
 1738 gatttttaatt tcgatcaaca aacatctcaa aatatcaaga atcagttagc ggaattaaac  
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 1918 gtagttaagg aggtcctatg agaagtaatt aattcgtcaa cagagggatt attgttaaat  
 1978 attgataagg atataagaaa aatattatca ggttatattg tagaaattga agatactgaa  
 2038 gggcttaaa aagtataaaa tgacagatat gatattgtga atatttctag ttacggcaa  
 2098 gatggaaaaa catttataga ttttaaaaaa tataatgata aattaccgtt atatatag  
 2158 aatcccaatt ataaggtaaa tgtatatgct gttactaaag aaacactat tattaatcct  
 2218 agtgagaatg gggatactag taccaacggg atcaagaaaa ttttaattct ttctaaaaa  
 2278 ggctatgaga taggataa

MAT-PA amino acid sequence

SEQ ID NO: 7

1 MEVKQENRLNNESES  
 17 SQQLLGYYFSDLNFOAPMVVTSSTTGDLSPSSSELENI PSENQY PQSAIWSGFIKVKK  
 75 SDEYTFATSADNHVTMWDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD  
 133 FKLYWTDSONKKEVTSSDNLQLPELKQKSSNSRKRST SAGPTVPDRDNDGIPDSLEV  
 191 EGYTVDVQKRTFLSPWISNIHEKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV  
 249 SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG  
 307 NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAE TMGLNTADTARLNAN  
 365 IRYVNTGTAPIYVNLPTTSLVLGKNQTLATIKAKENQLSQILAPNNYYPKONLAPIAL  
 423 NAQDDFSSTPITMNYNQFLEKTKQLRLD TDQVYGNIA TYNFENGRVRVD TGSNWSE  
 481 VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMLKEALKIAFGFNEPN  
 539 GNLYQGGKDITEFDNFQQTSQNIKNQLAEINATNYTVLDKIKLNAKMNI LIRDKR  
 597 FHYDRNIIAVGADESUVVKEAREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLK  
 655 EVINDRYDMLNISSLRQDGKTFIDFKKYN DKLPYISNPYKVNVYAVTKENTIINPS  
 679 ENGDTSTNGIKKILIFSKKGYEIG

MAT-PA gene sequence

SEQ ID NO: 2

1  
 34 gaatcagaat caagttccca ggggttacta atg gaagttaaac aggagaaccg gttattaaat  
 94 gcacccatgg tgggttacctc ttctactaca ggatactatt ttagtgattc gaattttcaa  
 154 gaaaatattc catcgaaaa ccaatatatt caatctgcta tttggtcagg atttatcaaa  
 214 gttagaaga gtgatgaata tacatttgct acttccgctg ataatcatgt aacaatgtgg  
 274 gtagatgacc aagaagtgt taataaagct tctaattcta acaaatcag attagaaaaa  
 334 ggaagattat atcaaataaa aattcaatat caacgagaaa atcctactga aaaaggattg  
 394 gatttcaagt tgtactggac cgattctcaa aataaaaaag aagtgatttc tagtgataac  
 454 ttacaattgc cagaattaaa acaaaaatct tcgaactcaa gaaaaaagcg aagtacaagt  
 414 gctggacctc cgttccaga ccgtgacaat gatggaatcc ctgattcatt agaggtagaa  
 474 ggatatacgg ttgatgtcaa aaataaaaaga acttttcttt caccatggat ttctaattt  
 534 catgaaaaa aaggattaac caaatataaa tcatctcctg aaaaatggag cagggttct  
 594 gatccgtaca gtgatttcga aaagggtaca ggacggattg ataagaatgt atcaccagag  
 654 gcaagacacc ccctgtggc agcttatccg attgtacatg tagatatgga gaattattt  
 714 ctctcaaaaa atgaggatca atccacacag aatactgata gtgaaacgag aacaataagt  
 774 aaaaatactt ctacaagtag gacacatact agtgaagtac atggaaatgc agaagtgc  
 834 gcgtcgttct ttgatattgg tgggagtgt tctgcaggat ttagtaattc gaattcaagt  
 894 acggtcgcaa ttgatcattc actatctcta gcaggggaaa gaacttgggc tgaacaatg  
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 1014 acggtcccaa tctacaacgt gttaccaacg acttcgtag tgttaggaaa aaatacaaca  
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 1194 ccaattacaa tgaattacaa tcaatttctt gagttagaaa aaacgaaaca attagatta  
 1254 gatacggatc aagtatatgg gaatatagca acatacaatt ttgaaaatgg aagagtggg  
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 1494 tttggattta acgaaccgaa tggaaaacta caatatcaag ggaaagacat aaccgaattt  
 1554 gatttttaatt tcgatcaaca aacatctcaa aatatcaaga atcagttagc ggaattaaac  
 1614 gcaactaaca tatatactgt attagataaa atcaaatata atgcaaaat gaattttta  
 1674 ataagagata aacgttttca ttatgataga aataacatag cagttggggc ggatgagtca  
 1734 gtagttaagg aggtctcatg agaagtaatt aattcgtcaa cagagggatt attgttaaat  
 1794 attgataagg atataagaaa aatattatca ggttatattg tagaaattga agatactgaa  
 1854 gggcttaaag aagttataaa tgacagatat gatattgtga atatttctag tttacggcaa  
 1914 gatggaaaaa catttataga ttttaaaaaa tataatgata aattaccgtt atatataggt  
 1974 aatcccaatt ataaggtaaa tgtatatgct gttactaaag aaacactat tattaatcct  
 2034 agtgagaatg gggatactag taccaacggg atcaagaaaa ttttaattct tctaaaaaa  
 2094 ggctatgaga taggataa

PA63 amino acid sequence

SEQ ID NO: 8

1 MSTSAGPTVPDRDNDGIPDSLEV  
 24 EGYTVDVVKIKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV  
 82 SPEARHPLVAAYPIVHVDMENTILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG  
 140 NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNAN  
 198 IRYVNTGTAPIYNVLPTTSLVLGKNQTLATIKAKENQLSQILAFNNYYPKONLAPIAL  
 256 NAQDDFSTPTITMNYNQFLELEKTKQLRLDQVYGNLATYNFENGVRVRVDTGSNWSE  
 314 VLPQIQETTARIIFNGKDLNLVERRIAAVNPSPLETTKPDMLKEALKIAPGFNEFN  
 372 GNLYQYQKIDITEFDNFDDQTSQNIKNQLAEINATNIYTVLDKIKLNKMNILIRDKR  
 430 FHYDRNNIAVGADESUVKEAHREVINSSTEGLLINIDKDIRKILSGYIVEIEDTEGLK  
 488 EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPYISNPNYKVNVAVTKENTLIINPS  
 546 ENGDTSTNGIKKILFFSKKGYEIG

PA63 gene sequence

SEQ ID NO: 4

1  
 13 gctggaccta cggttccaga ccgtgacaat gatggaatcc ctgattcatt agaggtagaa at gagtacaagt  
 73 ggatatacgg ttgatgtcaa aaataaaaga acttttcttt caccatggat ttctaataatt  
 133 catgaaaaaga aaggattaac caaatataaa tcatctcctg aaaaatggag cagggttctt  
 193 gatccgtaca gtgatttcga aaagggtaca ggacggattg ataagaatgt atcaccagag  
 253 gcaagacacc cccttggtggc agcttatccg attgtacatg tagatatgga gaatattatt  
 313 ctctcaaaaa atgaggatca atccacacag aatactgata gtgaaacgag aacaataagt  
 373 aaaaaatactt ctacaagtag gacacatact agtgaagtac atggaatgc agaagtgcatt  
 433 gcgtcgttct ttgatattgg tgggagtgtg tctgcaggat ttagtaattc gaattcaagt  
 493 acggtcgcga ttgatcattc actatctcta gcaggggaaa gaacttgggc tgaacaatg  
 553 ggtttaaata ccgctgatac agcaagatta aatgccaaata ttagatatgt aataactggg  
 613 acggctccaa tctacaacgt gttaccaacg acttcgttag tgttaggaaa aaatcaaaaa  
 673 ctccgcgacaa ttaaagctaa ggaaaaccaa ttaagtcaa tacttgacc taataattat  
 733 tatccttcta aaaacttggc gccaatcgca ttaaatgcac aagacgattt cagttctact  
 793 ccaattacaa tgaattacaa tcaatttctt gaggtagaaa aaacgaaaca attagatta  
 853 gatacggatc aagtatatgg gaatatagca acatacaatt ttgaaaatgg aagagttagg  
 913 gtggatacag gctcgaactg gagtgaagtg ttaccgcaaa ttcaagaaac aactgcacgt  
 973 atcattttta atggaagaaga tttaaatctg gttagaaaggc ggatagcggc ggtaatcct  
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 1093 tttggattta acgaaccgaa tggaaactta caatatcaag ggaagacat aaccgaattt  
 1153 gatttttaatt tcgatcaaca aacatctcaa aatatcaaga atcagttagc ggaattaaac  
 1213 gcaactaaca tatatactgt attagataaa atcaaatata atgcaaaaat gaataattta  
 1273 ataagagata aacgttttca ttatgataga aataacatag cagttggggc ggatgagtca  
 1333 gtagttaagg aggetcatag agaagtaatt aattcgtcaa cagagggatt attgttaaat  
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 1453 gggcttaaaag aagttataaa tgacagatat gatagtgtga atatttctag ttacggcaa  
 1513 gatggaaaaa catttataga ttttaaaaaa tataatgata aattaccgtt atataaagt  
 1573 aatcccaatt ataaggtaaa tgtatatgct gttactaaag aaacactat tattaatcct  
 1633 agtgagaatg gggatactag taccaacggg atcaagaaa ttttaattct ttctaataaa  
 1693 ggctatgaga taggataa

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